IB/05/00192

Sertifikaat

REPUBLIEK VAN SUID-AFRIKA

PATENT KANTOOR
DEPARTEMENT VAN HANDEL
EN NYWERHEID

Certificate

REPUBLIC OF SOUTH AFRICA

PATENT OFFICE
DEPARTMENT OF TRADE AND
INDUSTRY

Hiermee word gesertifiseer dat
This is to certify that

the documents annexed hereto are true copies of:

Application forms P.1 and P.3, provisional specification and drawings of South African Patent Application No. 2004/0685 as originally filed in the Republic of South Africa on 28 January 2004 in the name of CSIR for an invention entitled:" STABILIZATION OF PROTEINS".

Geteken te **PRETORIA** in die Republiek van Suid-Afrika, hierdie **22** dag van **March 2005**
Signed at in the Republic of South Africa, this day of

.....
Registrar of Patents

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

LATE LODGING OF DOCUMENTS

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SLIEK VAN SUID AFRIKA

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978
APPLICATION OR REQUEST TO THE REGISTRAR
(Section 30(6) - Regulation 38)

FORM P.5
(To be lodged in duplicate)

21	01	PATENT APPLICATION NO	22	LODGING DATE	43	A&A REF
		2004/0685		28 January 2004		V16101 GSK

71	FULL NAME(S) OF APPLICANTS

CSIR

74	ADDRESS FOR SERVICE

ADAMS & ADAMS, PRETORIA

The following documents not accompanying the application as lodged or required by the Registrar are hereby submitted:

	Request for ante-dating/delay of acceptance on Form P.4
	A copy of Form P.2 and the specification of RSA Patent Application No
X	A declaration and power of attorney on Form P.3
	An assignment of invention
	An assignment of priority rights
	Form PCT/IB 306
	Request on Form P014 for photocopies of papers received from WIPO
	One copy / two copies of the complete specification
	English Translation of the international application (in duplicate)
	English Translation of amendments annexed to the IPER (in duplicate)
	Verified English translation of the specification (in duplicate)
	Drawings of (sheets).
	Publication particulars and abstract (Form P.8 in duplicate)
	A copy of Figure of the drawings for the abstract
	Certified priority document(s). (State quantity) .....
	Verified translation of the priority document(s)

DATED THIS 19th DAY OF MAY 2004

REGISTRAR OF PATENTS DESIGNS, TRADE MARKS AND COPYRIGHT
2004 -05- 20
REGISTRATEUR VAN PATENTE, MODELLE, HANDELSMERKE EN OUTEURREG

ACKNOWLEDGEMENT OF LATE FILING OF DOCUMENTS

ADAMS & ADAMS
APPLICANTS PATENT ATTORNEYS

OFFICIAL DATE STAMP

REGISTRAR OF PATENTS

REGISTRAR OF PATENTS

A&A P205

ADAMS & ADAMS
PRETORIA

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978
DECLARATION AND POWER OF ATTORNEY
(Section 30 - Regulation 8, 22(i)(c) and 33)

FORM P.3

PATENT APPLICATION NO		
21	01	2004/0685

A&A REF: V16101 GSK

LODGING DATE	
22	28 JANUARY 2004

FULL NAME(S) OF APPLICANT(S)	
71	CSIR

FULL NAME(S) OF INVENTOR(S)	
72	1. MOOLMAN, Francis Sean 2. BRADY, Dean 3. SEWLALL, Avashnee Shamoarkesh 4. ROLFES, Heidi

EARLIEST PRIORITY CLAIMED	COUNTRY		NUMBER		DATE	
	33	---	31	---	32	---

NOTE: The country must be indicated by its International Abbreviation - see schedule 4 of the Regulations

TITLE OF INVENTION	
54	"STABILIZATION OF PROTEINS"

* I/~~We~~ ALETTA PETRONELLA SUSARAH TERBLANCHE

hereby declare that :-

* 1. ~~I/we am/are the applicant(s) mentioned above~~;

** 2. I/we have been authorized by the applicant(s) to make this declaration and have knowledge of the facts herein stated in the capacity of EXECUTIVE DIRECTOR of the applicant(s);

3. the inventor(s) of the abovementioned invention is/are the person(s) named above and the applicant(s) has/have acquired the right to apply by virtue of the provisions of Section 13 of Act 46 of 1988 ;

4. to the best of my/our knowledge and belief, if a patent is granted on the application, there will be no lawful ground for the revocation of the patent;

**** 5. ~~this is a convention application and the earliest application from which priority is claimed as set out above is the first application in a convention country in respect of the invention claimed in any of the claims~~; and

6. the partners and qualified staff of the firm of **ADAMS & ADAMS**, patent attorneys, are authorised, jointly and severally, with powers of substitution and revocation, to represent the applicant(s) in this application and to be the address for service of the applicant(s) while the application is pending and after a patent has been granted on the application.

SIGNED THIS 6th DAY OF April 2004

SIGNATURE(S)
(no legalization necessary)

* *In the case of application in the name of a company, partnership or firm, give full names of signatory/signatories, delete paragraph 1, and enter capacity of each signatory in paragraph 2.*
** *If the applicant is a natural person, delete paragraph 2.*
*** *If the right to apply is not by virtue of an assignment from the inventor(s), delete* "an assignment from the inventor(s)" *and give details of acquisition of right.*
**** *For non-convention applications, delete paragraph 5.*

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978
APPLICATION FOR A PATENT AND
ACKNOWLEDGEMENT OF RECEIPT
(Section 30(1) Regulation 22)

REPUBLIC OF SOUTH AFRICA
FORM P.1
(to be lodged in duplicate)
28.01.04 R 060.00

THE GRANT OF A PATENT IS HEREBY REQUESTED BY THE UNDERMENTIONED APPLICANT ON THE BASIS OF THE PRESENT APPLICATION FILED IN DUPLICATE

21	01	PATENT APPLICATION NO 2004/0685	A&A REF: JVW 840 GSK

71	FULL NAME(S) OF APPLICANT(S)

CSIR

ADDRESS(ES) OF APPLICANT(S)

Scientia, Pretoria, Gauteng, Republic of South Africa

54	TITLE OF INVENTION

"STABILIZATION OF PROTEINS"

Only the items marked with an "X" in the blocks below are applicable.

[ ] THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P.2. The earliest priority claimed is

Country: No: Date:

[ ] THE APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO 21 01

[ ] THIS APPLICATION IS A FRESH APPLICATION IN TERMS OF SECTION 37 AND BASED ON APPLICATION NO 21 01

THIS APPLICATION IS ACCOMPANIED BY:

X	A single copy of a provisional specification of 12 pages
X	Drawings of 3 sheets
	Publication particulars and abstract (Form P.8 in duplicate) (for complete only)
	A copy of Figure of the drawings (if any) for the abstract (for complete only)
	An assignment of invention
	Certified priority document(s). (State quantity)
	Translation of the priority document(s)
	An assignment of priority rights
	A copy of Form P.2 and the specification of RSA Patent Application No 21 01
X	Form P.2 in duplicate
	A declaration and power of attorney on Form P.3
	Request for ante-dating on Form P.4
	Request for classification on Form P.9
	Request for delay of acceptance on Form P.4
	Extra copy of informal drawings (for complete only)

74	ADDRESS FOR SERVICE: Adams & Adams, Pretoria

Dated this **28** day of **January 2004**

ADAMS & ADAMS
APPLICANTS PATENT ATTORNEYS

The duplicate will be returned to the applicant's address for service as proof of lodging but is not valid unless endorsed with official stamp

REGISTRAR OF PATENTS DESIGNS, TRADE MARKS AND COPYRIGHT
OFFICIAL DATE STAMP
2004 -01- 28
REGISTRATEUR VAN PATENTE, MODELLE, HANDELSMERKE EN OUTEURSREG

REGISTRAR OF PATENTS

A&A P201

A & A Ref No: V16101 GSK

ADAMS & ADAMS
PATENT ATTORNEYS
PRETORIA

FORM P6

REPUBLIC OF SOUTH AFRICA
Patents Act, 1978

PROVISIONAL SPECIFICATION

(Section 30 (1) - Regulation 27)

21	01	OFFICIAL APPLICATION NO

2004/0685

22	LODGING DATE

28 January 2004

71	FULL NAME(S) OF APPLICANT(S)

CSIR

72	FULL NAME(S) OF INVENTOR(S)

MOOLMAN, FRANCIS SEAN
BRADY, DEAN
SEWLALL, AVASHNEE SHAMPARKESH
ROLFES, HEIDI

54	TITLE OF INVENTION

"STABILIZATION OF PROTEINS"

THIS INVENTION relates to the stabilization of proteins. More particularly, it relates to a process for producing a stabilized protein structure.

Proteins in the form of enzymes are commonly required as catalysts in various industries, such as in chemical, pharmaceutical and cosmetic industries. However, unlike chemical catalysts, enzymes have limited application or shelf life due to their instability. Enzymes are extremely temperature and pH dependant, making their use in many processes difficult. In addition, soluble enzymes cannot be easily recovered from aqueous media, and enzyme activity generally decreases during storage or processing, limiting the application of enzymes as catalysts in chemical processing.

Commercial application of enzymes as catalysts can be enhanced by enzyme immobilization, which provides the dual advantages of increasing enzyme stability by making the enzymes more rigid, and increasing the overall size of the catalyst, making recovery simpler.

Immobilization of enzymes onto solid supports is therefore commonly practiced with the aim of stabilizing the enzymes and reducing costs by making them recyclable. However, immobilized enzymes display limitations, the most important being reduced enzyme activity per unit reactor volume due to only a small fraction of the immobilized volume constituting the active catalyst (enzyme). The Applicant is also aware of self-supported immobilized enzymes in the form of cross-linked enzyme crystals (CLEC) and cross-linked enzyme agglomerates (CLEA). Claims to increased activity have been made for both of these. In addition, CLEC and CLEA cross-linked enzymes are

stable in reaction media, and can be easily separated and recycled. CLEA appears to provide a less expensive and more efficient method compared to CLEC where time-consuming crystallization protocols are required. However, both CLEC and CLEA are limiting in that some active sites of the enzymes are not exposed, and hence processes utilizing either CLEA or CLEC would require excess enzyme catalyst (with an associated increased cost) for a particular function, to compensate for this.

It is thus an object of this invention to provide a process for producing a stabilized enzyme structure suitable for use as a catalyst, whereby these drawbacks are at least reduced.

Thus, according to the invention, there is provided a process for producing a stabilized protein structure, which process includes

providing an emulsion of droplets of a first liquid phase dispersed in a second liquid phase, with the one liquid phase being a hydrophilic phase and the other liquid phase being a hydrophobic phase which is immiscible with the hydrophilic phase, and with protein molecules being located at or within the interfacial boundaries of the droplets and the second liquid phase; and

cross-linking the protein molecules of the respective droplets, to form stabilized protein structures.

Since, in an emulsion, the droplets of the immiscible first liquid phase are normally spherical, the stabilized protein structures will thus normally be of hollow spherical form. In one embodiment of the invention, the individual structures may have openings so that the liquid phases can pass in or out of the structures. However, in another embodiment of the invention, the structures may be liquid impervious, ie they may be in the form of capsules, with the first liquid phase then being trapped inside the capsules. If such stabilized protein capsules are then used in a liquid reaction system, eg to catalyze the reaction system, they can easily be separated from the other components of the reaction system, eg by flotation, by selecting a first liquid phase having an appropriate density. However, when used in such a system, they need not necessarily only be separated by flotation since the fact that the

stabilized protein structures are self-supporting, means that they can easily be separated from the other components in the reaction system and recycled or re-used.

The protein molecules may, in particular, contain both hydrophilic and hydrophobic ends to ensure that they collect at the interfacial boundaries or interfaces of the droplets and the second liquid phase.

The protein may, in particular, be an enzyme. While the enzyme can be selected from enzyme classes such as Esterases, Proteases, Nitrilases, Nitrile hydratases, Oxynitrilases, Epoxide hydrolases, Halohydrin dehalogenases, Laccases, Penicillin amidases, Amino acylases, Ureases, Uricases, Lysozymes Asparaginases, Elastases, the enzyme is preferably lipase.

The lipase can be chosen from microbial, animal, or plant sources, including any one of the following: *Pseudomonas cepacia* lipase, *Pseudomonas fluorescens* lipase, *Pseudomonas alcaligenes* lipase *Candida rugosa* lipase, *Candida antarctica* lipase A, *Candida antarctica* lipase B, *Candida utilis* lipase, *Thermomyces lanuginosus* lipase, *Rhizomucor miehei* lipase, *Aspergillus niger* lipase, *Aspergillus oryzae* lipase, *Penicillium sp* lipase, *Mucor javanicus* lipase, *Mucor miehei* lipase, *Rhizopus arrhizus* lipase, *Rhizopus delemer* lipase, *Rhizopus japonicus* lipase, *Rhizopus niveus* lipase, Porcine Pancreatic lipase.

When lipase is used, the stabilized lipase structures may, in particular, be used in hydrolysis, acidolysis, alcoholysis, esterification, transesterification, interesterification, ammoniolysis, aminolysis, and perhydrolysis reactions. Other enzyme classes will be used in other reaction mechanisms particular to their function.

More particularly, the emulsion may be provided by dissolving the enzyme in the hydrophilic phase (herein also referred to as 'the water phase' or simply as 'W'), and forming the emulsion by mixing the enzyme containing hydrophilic

phase with the hydrophobic phase (herein also referred to as 'the oil phase' or simply as 'O'). Thus, the emulsion may be of the type O/W, ie oil or hydrophobic phase droplets in a continuous water or hydrophilic phase, W/O, ie water or hydrophilic phase droplets in a continuous oil or hydrophobic phase, O/W/O, W/O/W, or the like.

The process may further include selectively force precipitating the enzyme onto the droplet surfaces, for example, by increasing the concentration of a salt present in the water phase ('salting out').

The cross-linking of the protein molecules may be effected by means of a cross-linking agent. Thus, the process may include adding the cross-linking agent to the hydrophilic phase and/or to the hydrophobic phase and/or to the emulsion.

The process may further include separating the stabilized enzyme structures from the second liquid phase. The thus recovered stabilized protein structures may be washed, if desired, and thereafter dried, if also desired.

The process may further include, if desired, extracting the first liquid phase from the stabilized enzyme structures.

While the hydrophilic phase in which the enzymes are dissolved may comprise only water, it is believed that improved results may be achieved if it then includes a suitable buffer. The buffer should be selected to facilitate the cross-linking of the enzyme molecules, while ensuring enzyme stability. Thus, for example, the hydrophilic phase may comprise a phosphate buffered saline (PBS) solution with pH 7.8, a Tris-(hydroxymethyl)-aminomethane (TRIS) buffer-containing aqueous solution, or a KH_2PO_4/NaOH solution having a pH of 7.

Alternatively, the hydrophilic phase may comprise a polyethylene glycol (PEG). When a low molecular weight polyethylene glycol, such as PEG400 or PEG100, is used, it may be used on its own, ie the hydrophilic phase will then

consist of the low molecular weight polyethylene glycol. However, a higher molecular weight polyethylene glycol may optionally instead be used, with it then being dissolved in water to form the hydrophilic phase. When an isocyanate is used as the cross-linking agent in a water-in-oil emulsion, the cross-linking agent will react with the PEG as well as with the enzyme, leading to the formation of reinforced stabilized enzyme capsules that contain an enzyme incorporated membrane with an internal hydrogel support. This can advantageously improve the mechanical strength of the capsules, improving, for example, resistance against shear damage.

The water immiscible phase, ie the hydrophobic phase, may comprise an oil such as mineral, jojoba or avocado oil; a hydrocarbon such as decane, heptane, hexane or isododecane; an ester such as triglyceride, isopropyl palmitate or isopropyl myristate; or the like. It is believed that the emulsion used in the process of the invention will normally be in the form of a oil-in-water or O/W emulsion; however, as previously indicated, instead a water-in-oil or W/O oil-in-water-in-oil, ie O/W/O, or water-in-oil-in-water, ie W/O/W, emulsion can be used. Thus, for example, when the enzyme is lipase, a water-in-oil emulsion can be used to ensure that most of the lipase active sites, which are hydrophobic, are oriented outwardly, thus increasing the total effective activity of the structures.

Furthermore, when a water-in-oil emulsion is used, a second enzyme can advantageously be dissolved in the aqueous or hydrophilic phase. If this second enzyme also has the ability to accumulate at the droplet/second liquid phase interfaces, the resultant cross-linked enzyme structures will contain both enzymes. Alternatively, if the second enzyme is selected so that it does not accumulate at the interfaces, a cross-linked enzyme structure will result with one enzyme being a major component of the structure, while the second enzyme is encapsulated or contained inside the structure. Such a combination enzyme structure can advantageously be used, for example, to catalyze multiple reactions in a single reaction step.

In a particular embodiment of the invention, a triglyceride, which is hydrolysable by lipase, may be used as the hydrophobic or oil phase, with an O/W emulsion being formed; the dispersed or oil phase, ie the triglyceride, contained within the stabilized cross-linked structures or spheres is hydrolyzed by the lipase during and after the cross-linking reaction. The hydrolyzed products are generally water-soluble, and can thus readily be leached out, thereby minimizing or reducing the number of processing steps required to produce the stabilized structures.

In yet another embodiment of the invention, an initial O/W emulsion can be formed. In doing so, a certain degree of purification of the lipase takes place, since impurities present therein will also tend to collect at the interfacial boundaries, albeit to a lesser extent. The process may then include, before effecting the cross-linking, centrifuging the emulsion and separating a concentrated emulsion from a dilute water phase. Thereafter, a further O/W emulsion can be formed, using the concentrated emulsion. This step can, if desired, be repeated one or more times, to increase lipase purity. After the final such purification step, the emulsion may then be inverted to form a W/O emulsion, by the addition of surfactants with lower HLB values, which may be in the range of 3-10, more preferably 4-6. This ensures preferential orientation of the lipase active sites towards the outside of the dispersed phase droplets. Thereafter, cross-linking of the lipase as hereinbefore described, can be effected.

To impart specific properties to the stabilized enzyme structures, a modifier may be added to the hydrophilic phase and/or to the hydrophobic phase and/or to the emulsion. One or more of the following modifiers can be added in this fashion: a surfactant, a precipitator and an additive.

A surfactant may be used when it is desired to impart enhanced enzyme activity (as regards its use in a subsequent catalyzed reaction), and improved emulsion stability. The surfactant may be anionic, cationic, non-ionic, zwitterionic, polymeric, or mixtures of two or more of these. When an anionic surfactant is used, it may be an alkyl sulphate such as sodium lauryl sulphate

or sodium laureth sulphate, or an alkyl ether sulphate. When a cationic surfactant is used, it may be centrimonium chloride. When a non-ionic surfactant is used, it may be an ethoxylated alkyl phenol such as polyoxyethylene(10) iso-octylcyclohexyl ether (Triton X100) or polyoxyethylene(9) nonylphenyl ether (Nonoxynol-9). When a zwitterionic or amphiphillic surfactant is used, it may be decyl betaine. When a polymeric surfactant is used, it may be an ethylene oxide-propylene oxide-ethylene oxide triblock copolymer, also known as a poloxamer, such as that available under the trade name Pluronic from BASF, or it may be a propylene oxide-ethylene oxide-propylene oxide triblock copolymer, also known as a meroxapol.

A precipitator can be used when it is desired to precipitate the enzyme onto the emulsion interfaces. The precipitator, when present, may be an inorganic salt such as ammonium sulphate; an inorganic solvent such as 1,2-dimethylethane or acetone; or a dissolved polymer.

Additives or adjuvants will be used to impart desired properties to the emulsion and/or to the stabilized enzyme structures. Properties that can be modified by use of such additives include pH, by using, for example, a buffer; ionic strength, by using, for example, salts; viscosity, by using, for example, PEG; agglomeration tendency, by using, for example, a surfactant possessing steric hindrance properties; and zeta potential, by using, for example, an anionic surfactant.

The cross-linking agent is a multifunctional reagent, ie a molecule having two or more functional groups or reactive sites which can react with groups on the enzyme to form a cross-linked macromolecule, ie the stabilized structure. The cross-linking agent may be selected from the following: an isocyanate such as hexamethylene diisocyanate or toluene diisocyanate; an aldehyde such as glutaraldehyde, succinaldehyde and glyoxal; an epoxide; an anhydride; or the like.

Separation of the stabilized enzyme structures from the second liquid phase may be effected by means of flotation, filtration, centrifugation or the like.

Drying of the stabilized protein structures may be effected by means of spray drying, vacuum drying or lyophilization (freeze drying).

When it is desired to extract the first liquid phase (normally the oil phase) from the stabilized protein capsules, this may be effected by contacting the stabilized protein capsules with an organic solvent capable of dissolving the first liquid phase, or by contacting the capsules with a mixture of a suitable surfactant in water. Alternatively, the first liquid phase can be extracted by supercritical fluid extraction. The fluid is then preferably supercritical carbon dioxide. The critical point for carbon dioxide (31.2°C and 73.8 bar) is sufficiently low so that the extraction process will not damage the stabilized protein structure.

The invention will now be described in more detail with reference to the following non-limiting and non-optimized examples and the accompanying drawings.

In the drawings,

FIGURE 1 is an optical microscope picture of cross-linked lipase capsules prepared in accordance with Example 1;

FIGURE 2 is a particle size distribution of the cross-linked lipase capsules prepared in Example 1; and

FIGURE 3 is a photograph showing cakes of dried enzyme capsules (from a water-in-oil emulsion) after extracting the oil phase using supercritical carbon dioxide, in accordance with Example 3.

EXAMPLE 1

Cross-linked or stabilized enzyme spheres (structures) from oil-in-water emulsion

1 g of lipase Amano AK was added to 195 g PBS (pH 7.8) and 5 g mineral oil (Castrol). This blend was then homogenized for 5 minutes using a Silverson

L4R laboratory rotor-stator homogenizer at 6000 rpm. 1.5 g of hexamethylene di-isocyanate (Merck Schuchardt) was added to the emulsion. The emulsion was then stirred at room temperature for 2 hours. The cross-linked enzyme structures were then recovered by filtration using 0.45 µm filter paper and washed 5 times with 50 ml of PBS each time (total 250 ml PBS). Figure 1 shows typical stabilized enzyme spheres or structures obtained according to the method. Particle sizes were determined using laser light scattering (Malvern Mastersizer 2000), and an average Sauter mean diameter of 49.4 µm was obtained (see Fig. 2).

The activity of the stabilized enzyme (lipase) structures was determined using a p-Nitrophenylacetate assay method as described by Vorderwülbecke, T., Kieslich, K. & Erdmann, H. (1992). 'Comparison of lipases by different assays', Enzyme Microb. Technol., 14, 631-639; and López-Serrano P., Cao L., van Rantwijk & Sheldon R.A. (2002). 'Cross-linked enzyme aggregates with enhanced activity : application to lipases', Biotechnology Letters., 24, 1379-1383.

This assay measures the release of ρ-nitrophenol from a ρ-nitrophenyl ester of a fatty acid. The reaction is done at pH 7.4 at 37°C and the liberated ρ-nitrophenol is measured at 410nm. The activity obtained was 63 U/g lipase, where U is µmol/min.

EXAMPLE 2

Glutaraldehyde as cross-linker

Example 1 was repeated, except that the cross-linking agent used was glutaraldehyde.

EXAMPLE 3

Cross-linked enzyme spheres from water-in-oil emulsion

Example 1 was repeated, except that a water-in-oil emulsion was generated, using Span 20 and Tween 20 as surfactants (total surfactant level was 20% by weight of dispersed phase, with ratio between low HLB surfactant (Span

20) and high HLB surfactant (Tween 20) the same as the ratio between hydrophobic (mineral oil) and hydrophilic (aqueous buffer) phases). After cross-linking, the emulsion was centrifuged in a Jouan C1000-S5L centrifuge at 10 000 rpm for 5 minutes. This caused the emulsion to sediment. The bottom layer (containing the cross-linked enzyme spheres) was then recovered by decantation. Oil was then extracted from this layer using supercritical carbon dioxide at 350 bar for 30 minutes. Figure 3 shows the dried enzyme capsule material recovered from the supercritical fluid extractor.

Subsequently, the powder was resuspended in water, homogenized at 5000 rpm for 5 minutes using a Silverson L4R laboratory homogenizer, and redried. The activity obtained was 26 U/g lipase.

It is believed that the process of the present invention, which provides the stabilized enzyme hollow spherical structures, provides the following advantages when the structures are subsequently used to catalyze reactions:

1. Maximum exposed surface area of catalyst (spherical, hollow capsules).
2. Buoyancy of catalyst can be controlled, eg, floating particles could be separated from the reaction medium with ease.
3. The mean size (diameter) of the immobilized enzyme particle formed can be controlled by controlling the size distribution of the emulsion.
4. Through use of the natural self-orientation of many lipases at solvent interfaces, the immobilized enzyme sphere may be generated in a controlled manner so as to orientate the majority of active sites either towards the lumen or externally as required.
5. Due to the presence of a hydrophilic/ hydrophobic interface, the lipase will be immobilized in the active form.

The invention thus provides a method of stabilizing a protein, and in particular an enzyme, by means of cross-linking, using emulsions as a vehicle therefore. The invention also relates to exposing maximum surface area of enzyme per unit volume of the structure, for subsequent reaction when the structure is

used as a catalyst. Additionally, the stabilized enzyme structures are easily recyclable, less expensive than known self-supporting enzymes, and will find widespread application as catalysts in various processes.

Additionally, due to the selective orientation of some proteins (such as lipases) at the hydrophilic/hydrophobic phase interface, they will be concentrated there. So this method, when applied in the example of an oil in water emulsion, will simultaneously purify the desired lipase from a crude cell lysate. The same would be true of other proteins with external hydrophobic regions, including many membrane-associated enzymes.

The cross-linking of lipases at the phase interface will fix them in the activated (lid open) state.

The use of oil-in-water emulsions will permit mono-layer lipase spheres, thereby providing a cross-linking method that provides the maximum surface area to protein mass.

The use of water-in-oil emulsions would allow for denser, multi-layered enzyme spheres.

This form of enzyme immobilization allows for enzyme recovery and reactor recycling.

DATED THIS 28TH DAY OF JANUARY 2004.

ADAMS & ADAMS
APPLICANTS PATENT ATTORNEYS

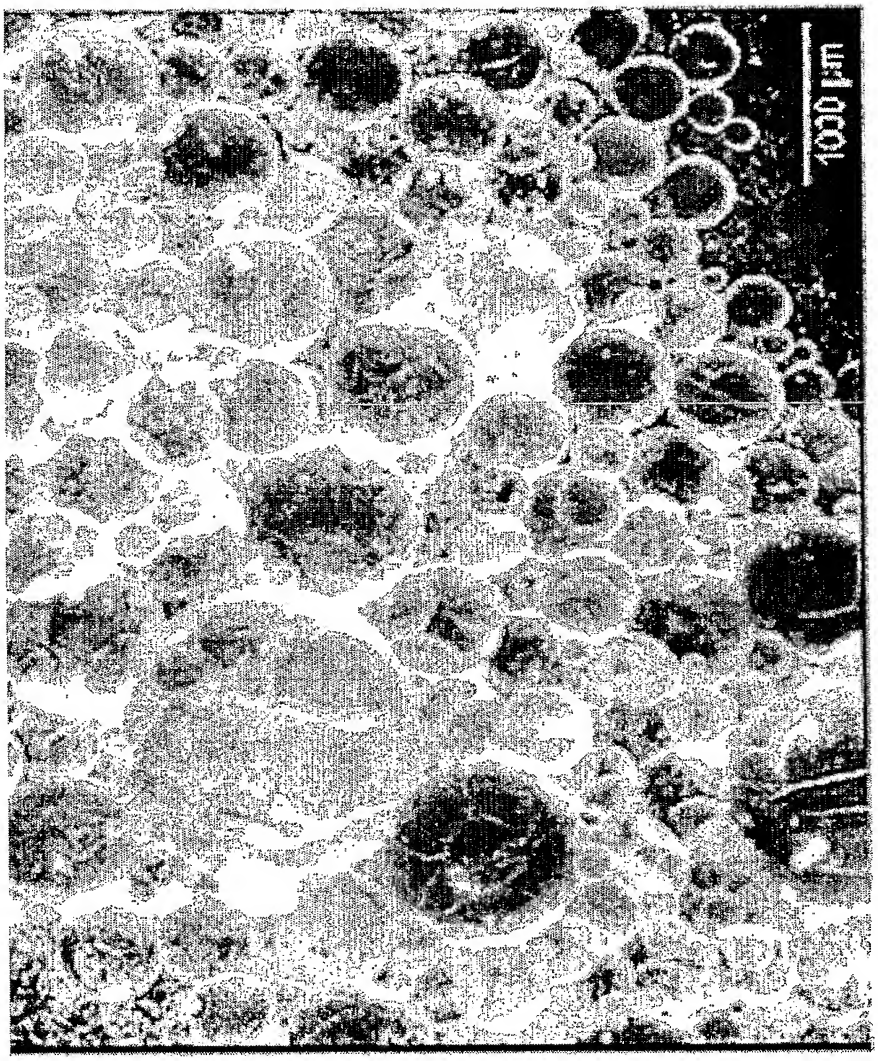

Fig 1

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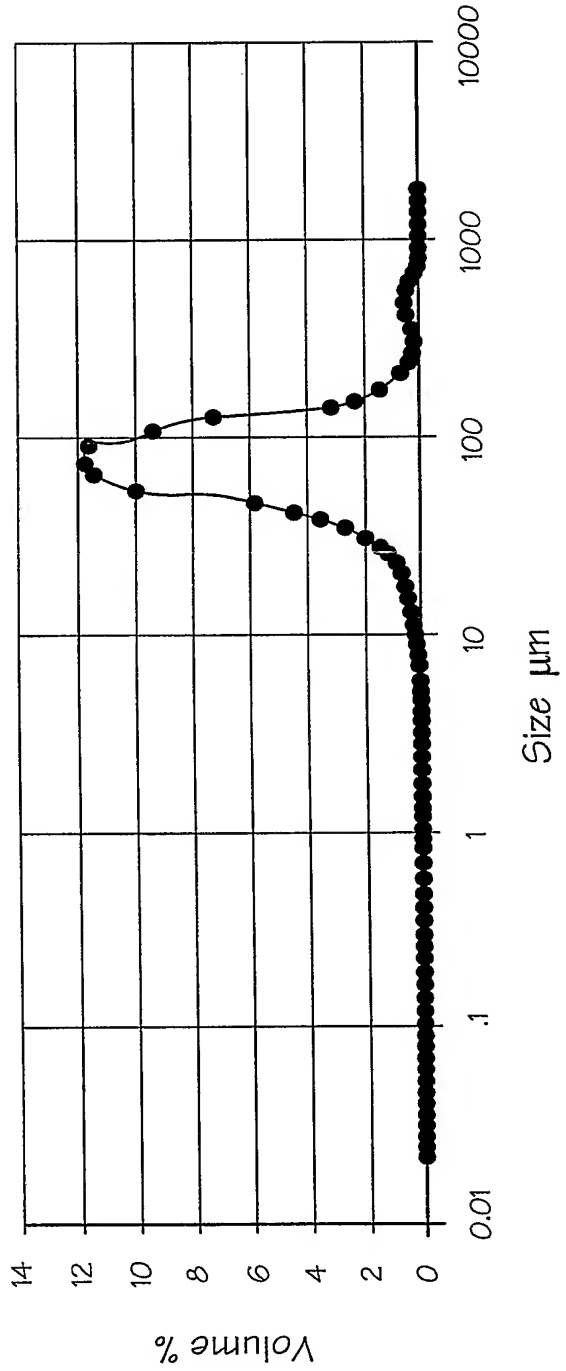

Fig 2

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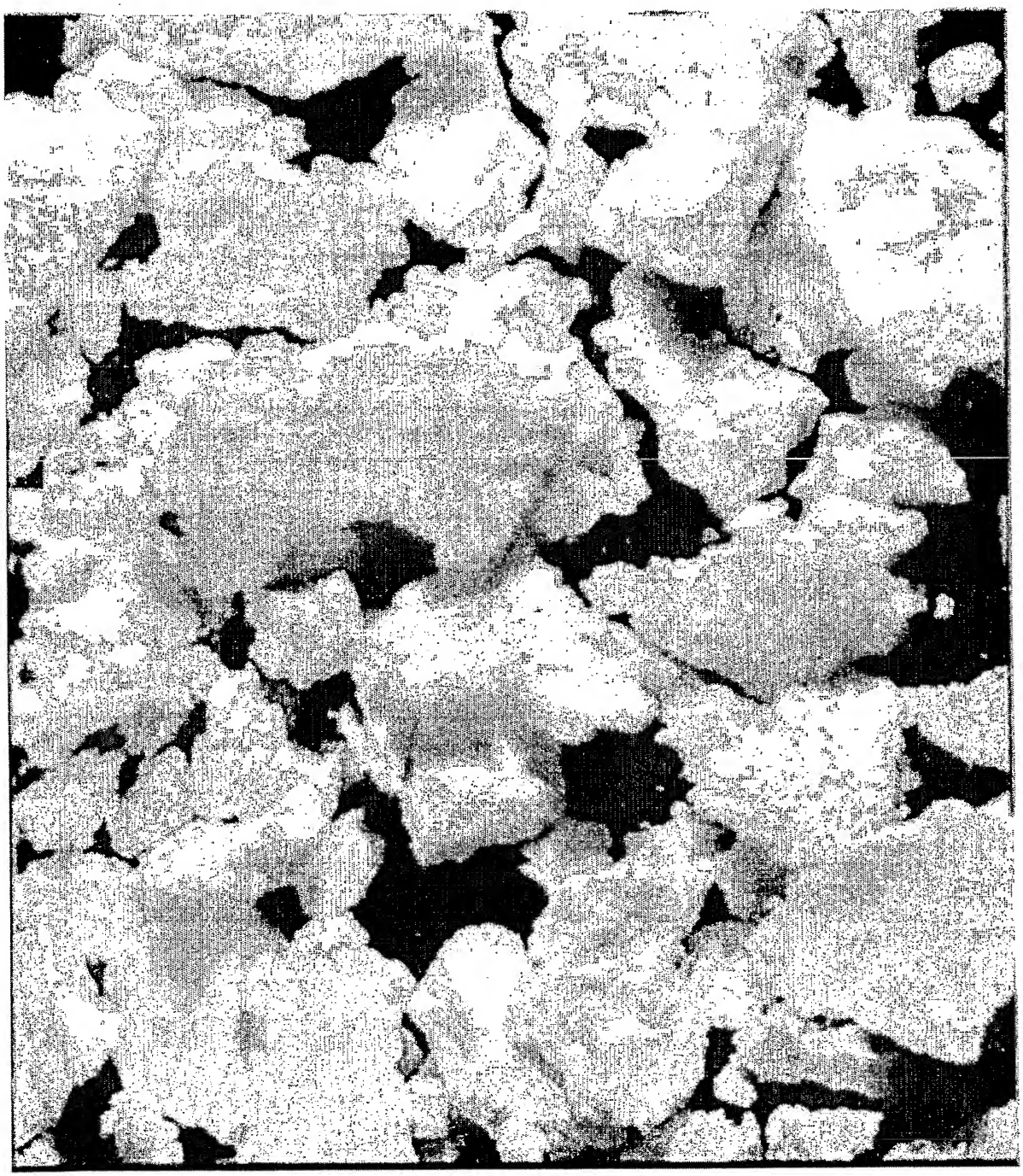

Fig 3

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APPLICANTS PATENT ATTORNEYS